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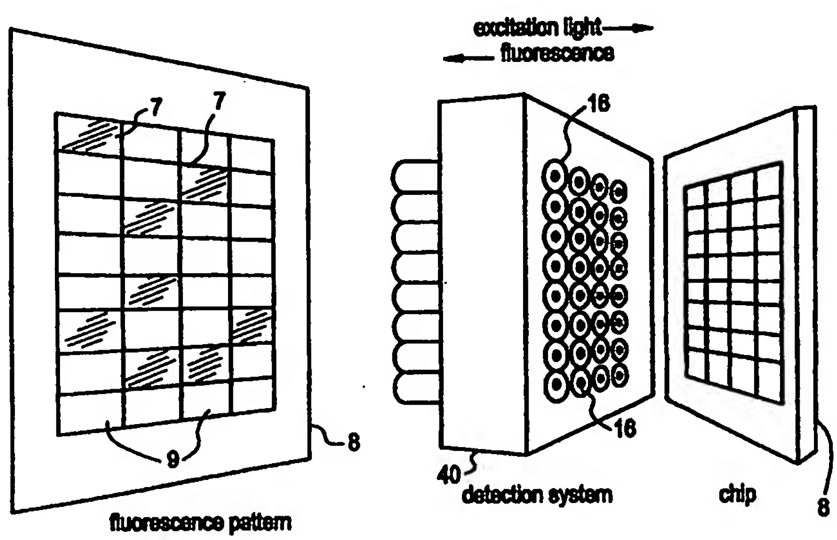

(57) Abstract

Method and apparatus for use in the identification of microorganisms, proteins and peptides in which a microorganism containing sample is contacted with a sensor chip having on a surface thereof a patterned array of a plurality of sections, each section having bonded thereto a ligand capable of binding a microorganism, protein or peptide. A number of different ligands are bonded to the various sections of the sensor chip, and thus serve to capture the microorganism, protein or peptide. Electromagnetic radiation is directed to the surface to ascertain which of the sections contains a microorganism, protein or peptide captured thereon, and then the microorganism, protein or peptide is identified as a function of one or more different ligands having a microorganism, protein or peptide bonded thereto.

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TAXONOMIC IDENTIFICATION OF MICROORGANISMS, PROTEINS AND PEPTIDES INVOLVED IN VERTEBRATE DISEASE STATES

Background Of The Invention

The present invention relates to a method and apparatus for the taxonomic identification of microorganisms, and more particularly to the taxonomic identification of pathogenic microorganisms.

Pathogenic microorganisms, particularly pathogenic bacteria which either occur naturally or which have acquired virulence factors, are responsible for many of the diseases which plague mankind. Many of these bacteria have been proposed as biowarfare agents in the past. In addition, there is also the risk and likelihood that nonpathogenic microbes could also be used after genetic manipulation (*e.g.*, *Escherichia coli* harboring the cholera toxin).

Typical pathogenic bacteria include those responsible for botulism, bubonic plague, cholera, diphtheria, dysentery, leprosy, meningitis, scarlet fever, syphilis and tuberculosis, to mention a few. During the last several decades, the public perception has been one of near indifference in industrialized

nations, principally because of successes that have been achieved in combating these diseases using antibiotic therapy. However, bacteria are becoming alarmingly resistant to antibiotics. In addition, there have been recent revelations of new roles that bacteria perform in human diseases such as Heliobacteria phylori as a causitive agent of peptic ulcers, Burkholderia cepaccia as a new pulmonary pathogen and Chlamydia pneumoniae as a possible trigger of coronary heart disease. Apart from those pathogens, various socioeconomic changes are similarly contributing to the worldwide rise in food-borne infections by bacteria such as Escherichia coli, Salmonella spp., Vibrio spp., and Campylobacter jejuni.

Potential infections are also important considerations in battlefield medicine. A number of bacterial pathogens, including Bacilis anthracis and Yersininia pestis and their exotoxins, have been used as weapons in the past. And, as noted, there is always the increasing risk that nonpathogenic microbes can be engineered to be pathogenic and employed as biowarfare agents.

Pathogenic microorganisms are also of concern to the livestock and poultry industries as well as wildlife management. For example, Brucella abortis causes the spontaneous abortion of

calves in cattle. Water supplies contaminated with exotoxin-producing microorganisms have been implicated in the deaths of bird, fish and mammal populations. More recently, mad cow disease has been traced to the oral transmission of a proteinaceous particle not retained by filters. Thus, there is clearly a need for the rapid and inexpensive techniques to conduct field assays for toxic proteins and pathogenic microorganisms that plague animals as well as humans.

As a general proposition, bacterial contamination can be detected by ordinary light microscopy. This technique, however, is only of limited taxonomic value. The investigation and quantitation of areas greater than microns in size are difficult and time consuming. Many commercially available systems rely on the growth of cultures of bacteria to obtain sufficiently large samples (outgrowth) for the subsequent application of differential metabolic tests for species (genus) identification. However, techniques requiring bacterial outgrowth may fail to detect viable but nonculturable cells. To the contrary, the growth media employed may favor the growth of bacteria with specific phenotypes.

More sensitive and more rapid typing schemes are described in "Strategies to Accelerate the Applicability of Gene

Amplification Protocols for Pathogen Detection in Meat and Meat Products" by S. Pillai and S.C. Ricke and "Molecular Approaches for Environmental Monitoring of Microorganisms" by R.M. Atlas, G. Sayle, R.S. Burlage, and A.K. Bej. Those techniques employ the polymeric chain reaction (PCR) for amplification of bacterial DNA or RNA, followed by nucleic acid sequencing to detect the presence of a particular bacterial species. Such general amplification and sequencing techniques require technical expertise and are not easily adaptable outside of specialized laboratory conditions. Moreover, such PCR methodology cannot indicate whether the target bacterium was viable; this technique provides a positive analysis whenever an intact target nucleic acid sequence is detected.

Another approach utilizes immunochemical capture as described in "The Use of Immonological Methods to Detect and Identify Bacteria in the Environment" by M. Schlotter, B. Assmus and A. Hartmann Biotech. Adv. 13, 75, followed by optical detection of the captured cells. The most popular immunoassay method, enzyme-length immunosorbent assay (ELISA), has a detection limit of several hundred cells. That is well below the $I.D._{50}$ of extremely infectious bacteria such as Shigella flexneri. Piezoelectric detection techniques, such as those described by "A Piezoelectric Biosensor for Listeria

Monocytogenes" by M.B. Jacobs, R.M. Carter, G.J. Lubrano and G.G. Guilbault, are even less sensitive having a detection limitation of about 5 x 10^5 cells. A recent report entitled "Biosensor Based on Force Microscope Technology" by D.R. Baselt, G.U. Lee and R.J. Colton describes the use of an atomic force microscope (AFM) to detect immunocaptured cells. Like other immunoassay techniques, viable cells cannot be discriminated from dead cells because cell capture and detection is predicated on the presence of an intact bacterial antigen. Immunoassays are also presently used in the trace analysis of peptides and proteins.

Moreover, the prior art has made extensive use of immobilized antibodies in peptide/protein/microorganism capture. Those techniques likewise involve significant problems because the antibodies employed are very sensitive to variations in pH, ionic strength and temperature. Antibodies are likewise susceptible to degradation by a host of proteolytic enzymes in "dirty" samples. In addition, the density of antibody molecules supported on surfaces (e.g., microwell plates or magnetic beads) is not as high as is frequently necessary.

Medical and military considerations call for better toxin and pathogen detection technologies. Real-time assessment of battlefield contamination by a remote sensing unit is

necessary to permit and facilitate rapid diagnosis to permit appropriate counter-measures. A microbe/toxic protein sensor useful in such situation requires the ability to globally discriminate between pathogens and nonpathogens as well as discriminate between viable cells (including spores) and dead cells. In addition, such techniques require high sensitivity when ten or fewer cells are present and analysis that can be completed in the field in less than 15 minutes. In cases such as this, even in pyrolysis, followed by mass spectral analysis of volatile cell components (e.g., fatty acids) would be extremely difficult and hence impractical for routine assays. Such techniques should be able to recognize pathogens and provide some assessment of strain virulence or toxigenicity.

In copending application Serial No. 559,043, filed June 3, 1996, the disclosure of which is incorporated herein by reference, there is described a method and apparatus for sensing the presence of microbes on a non-living surface which is particularly well-suited to detect the presence of microbes in meat, poultry and like food products. In accordance with the system described in the foregoing copending application, microbes which may be present on non-living surfaces such as meat and poultry are subjected to electromagnetic energy having wavelengths greater than about 350 nm. The electromagnetic

radiation excites the microbial cells present on the surface to emit electromagnetic energy (i.e., fluoresce) having a wavelength greater than that of the excitation wavelength. Any microbial cells present on the surface containing reduced pyridine nucleotides such as nicotinamide adenine dinucleotide (NADH) will emit a characteristic fluorescence signal. The presence of living microbial cells is determined by sensing both the fluorescence from the cell respiration and electromagnetic radiation reflected or scattered by the surface.

While the system disclosed in the foregoing application represents a significant advance in the art in detecting the presence of living microbial cells, it cannot be used as such to taxonomically evaluate the microbes present on the surface, or identify proteinaceous toxins or peptide hormones of pathophysiological importance to vertebrate animals, including humans, livestock, poultry and wildlife.

It is accordingly an object of the present invention to provide a method and apparatus for taxonomically evaluating microbes, proteins or peptides which overcome the foregoing disadvantages.

It is a more specific object of the invention to

provide a method and apparatus for taxonomically evaluating microbes, proteins and peptides which have the capability of discriminating between pathogens and nonpathogens and viable cells from dead cells and can be likewise used to identify proteins or peptides.

It is yet another object of the invention to provide a method and apparatus for taxonomically evaluating microbial cells characterized by high sensitivity (ten to one hundred cells) and which can taxonomically identify microbes under field conditions and can likewise be used to identify trace amounts of proteins or peptides.

Summary Of The Invention

The concepts of the present invention reside in a method and apparatus for the taxonomic identification of microorganisms in which microbes are captured through the binding of microbial receptors to specific ligands tethered to a surface, and the electromagnetic radiation is used to determine the presence of metabolites or other characteristic biomolecules for the detection of the presence of the captured microorganisms in accordance with the practice of the invention, a microorganism-containing sample is contacted with a sensor chip,

the sensor chip having a patterned area on its surface containing a plurality of sections, with each section having bonded thereto a ligand capable of bonding to a specific microbial receptor. The receptor may be, for example, a protein residing in the outer membrane of the microbial cell, pilus or flagellum which is exposed to the aqueous environment surrounding a cell. In accordance with the concepts of the present invention, the same receptor likewise forms the basis for the detection of peptides and proteins of pathological interest.

Electromagnetic radiation is directed onto the surface of the sensor chip to excite sections of the sensor chip to determine which of the sections of the sensor chip contain a microorganism binding to the ligand on that particular section. By determining which sections have a microorganism bound to a ligand, it is possible to taxonomically identify the microorganism contained in the sample as a function of the combination of different ligands which have a microbe bound thereto.

Thus, the method of the present invention does not depend on classical antigen-antibody recognition. On the contrary, the concepts of the present invention make use of

relatively inexpensive reagents in the capture of microorganisms, peptides or proteins contained in the sample.

The sensor chip employed in the practice of the present invention are preferably formed from a suitable support material such as glass or non-reactive plastic substrates such as polystyrene and polymethylmethacrylate. The sensor chip is formed of a patterned array defining a plurality of sections on the surface of the sensor chip, and each section has bonded thereto a different ligand capable of molecularly recognizing a specific peptide, protein or microbial receptor and hence the microbe itself. The ligand for pathogen/peptide/protein capture bonded to the surface of the sensor chip can and should be varied. In general, such ligands may be characterized as heme, siderophores, oligosaccharides and anti-adhesion peptides capable of capturing a wide variety of microorganisms, toxic proteins and peptides. Those ligands can thus be immobilized or bonded to the surface of the sensor chip by means of organic coupling agents having the capability of reacting with the surface of the sensor chip itself and also having the capability of reacting with the ligands whereby the coupling agent establishes a chemical bond or "tether" between the surface of the sensor chip and the ligand capable of reaction with a variety of different microorganisms, proteins and/or peptides.

Particularly useful in the bonding of the ligands to glass sensor chip substrates are the organosilanes having 1-3 readily hydrolyzable groups attached directly to the silicon atom and a functional organic group also attached to the silicon atom, the functional group being capable of reaction with the ligand.

In the preferred practice of the invention, the patterned array of the sensor chip is preferably positioned on the surface thereof in a pattern such as rows of sections. Each one of the sections, in the preferred practice of the invention, is exposed to electromagnetic radiation to excite captured protein, peptide or biomolecule present in the captured microbes having characteristic emission fluorescence. That fluorescence can then be detected by a suitable apparatus for detecting electromagnetic radiation and converting that radiation into an electrical signal as an indication of whether or not a particular section has a microorganism, protein or peptide bonded to the ligand for that section. In the preferred practice of the invention, the probe, which may be a probe like that described in the foregoing copending application, can be sequentially positioned to direct electromagnetic radiation to each of the sections in turn. Alternatively, each section of the sensor chip can be scanned simultaneously. The output of the fluorescence detector can then be converted to an electrical

signal indicative of those sections having a microorganism, protein or peptide bonded thereto. The microorganism, protein or peptide present can thus be identified by examining which of the sections have captured the species of interest and which have not.

Thus, the present invention can be rapidly used to identify microorganisms without the need for growing a culture of the microorganism and then microscopically examining the culture thus produced. Likewise, low levels of toxic proteins or peptide hormones can similarly be identified. It is also unnecessary to employ enzymes or antibodies in the capture of microbial metabolites as is often used in the prior art.

Brief Description Of The Drawings

Fig. 1 is a schematic illustration showing the sensor chip employed in the practice of the invention, illustrating the different sections contained on the face thereof having different ligands attached to each section.

Fig. 2 is a schematic illustration of a multiple element detection system which can e used in the practice of the present invention.

Fig. 3 is a graph illustrating the fluorescence excitation (EX) and emission (EM) spectrum of a sample of meat with and without E coli contamination and meat with fat having no E coli contamination. Contamination was approximately 10^3 cells/cm^2.

Fig. 4 is a graph illustrating fluorescence excitation (EX) and emission (EM) spectrum of tryptophan (Trp), tyrosine (Tyr) and calcium dipicolinate (DP) in Bacillus cereus spores.

Fig. 5 illustrates schematically a system embodying the present invention for scanning a sensor chip in the practice of the invention.

Detailed Description Of The Invention

In the practice of the present invention, a sample containing an unknown analyte microorganism, protein or peptide is first contacted with the sensor chip. The sensor chip is illustrated in Figure 1 of the drawings and is formed of a substrate 8 such as a glass slide having a series of sections formed thereon, each of which having a series of sections 1 through 70 on the surface thereof. Each section has a different ligand bonded thereto so as to be capable of binding to specific

analytes. The ligands are capable of binding to the analyte for capture and the presence of the captured analyte is detected using the fluorescence detection system disclosed and claimed in copending application Serial No. 659,043, filed June 3, 1996. Thus, the ligand of each of the sections of the sensor chip 8 has the capability of capturing a specific microorganism, peptide or protein.

In the preferred practice of the invention, the ligands used in the present invention are taken from the group of heme compounds, siderophores, oligosaccharides and peptides.

As is well known to those skilled in the art, animal pathogens generally possess heme uptake capability, and thus heme compounds can be used to capture a number of pathogenic species. In addition to heme compounds, other ligands in the form of high-affinity iron chelators, generally referred to as siderophores, can also be used to capture many strains of pathogenic bacteria. Included among such siderophores are alcaligin, mycobactins, pyochelin, staphyloferrin, vibriobactin and yersiniabactin.

In addition to heme compounds and siderophores, eukaryotic surface epitopes (peptides or carbohydrates) which

are recognized by microbial cell receptors, can likewise be used as ligands in the practice of the present invention. These ligands include commercially available oligosaccharides as well as those available by chemical synthesis. Other oligosaccharides and their affinity to pathogens from various microorganisms are described by Karlsson "Microbial Recognition of Target Cell Glycoconjugates", Structural Biology, 1995, 5: 622,635, the disclosure of which is incorporated herein by reference.

The characteristics of a number of bacterial species along with the diseases caused by such bacteria and their binding characteristics with siderophores, oligosaccharides and hemin are set forth in Table I. These characteristics can be used in the capture and identification of such species.

Peptide ligands can be produced by affinity panning of libraries of oligopeptides displayed on bacteriophages or on *Escherichia coli* flagella. Such ligands are useful in the capture of soluble proteins and peptide hormones as well as microorganisms.

Table 1

Bacterial Species	Disease Caused	Siderophore Binding?	Oligosaccharide Binding?	Hemin Binding?	Exotoxin Produced?
Bacillus anthracis	anthrax	unknown	unknown	unknown	anthrax toxin
Bordetella pertussis	whooping cough	alcaligin, others	N-Acetyl-glucosamine	Yes	pertussis toxin
Clostridium botulinum	botulism	unknown	unknown	Yes	botulinum toxin
Clostridium perfringens	gas gangrene	unknown	unknown	unknown	α-toxin
Clostridium tetani	tetanus	unknown	unknown	unknown	tetanus toxin
Corynebacterium diphtheriae	diphtheria	aerobactin	unknown	unknown	diphtheria toxin
Escherichia coli 0157:H7	numerous infections	many	globobiose, others	Yes	Shiga-like toxin
Haemophilus influenzae	meningitis	enterobactin	GalNAcβ(1-4)Gal, others	Yes	unknown
Helicobacter pylori	gastric ulcers	unknown	a mucosal oligosaccharide	unknown	vacuolating cytotoxin A
Klebsiella pneumoniae	numerous infections	many	GalNAcβ(1-4)Gal, others	Yes	unknown

Table 1 (cont.)

Bacterial Species	Disease Caused	Siderophore Binding?	Oligosaccharide Binding?	Hemin Binding?	Exotoxin Produced?
Mycobacterium tuberculosis	tuberculosis	mycobactin T	unknown	unknown	unknown
Neisseria meningitidis	meningitis	many	unknown	Yes	unknown
Pseudomonas aeruginosa	numerous infections	pyochelin, others	asialo G_{M1}, others	Yes	exotoxin A, others
Salmonella typhi	typhoid fever	many	unknown	Yes	Yes
Serratia marescens	numerous infections	aerobactin, ferrioxamine B	Yes	Yes	Yes
Shigella dysenteriae	dysentery	enterobactin, aerobactin	Yes	Yes	Shiga toxin
Staphylococcus aureus	numerous infections	staphyloferrin, others	GalNAcβ(1-4)Gal	Yes	several superantigens
Streptococcus pneumoniae	pneumonia, meningitis	unknown	GlcNAcetyl-(1-3)Gal, others	Yes	Yes
Vibrio cholerae	cholera	vibriobactin, others	Yes	Yes	cholera toxin
Yersinia pestis	bubonic plague	yersiniabactin	unknown	Yes	YopE

Toxins that contain at least one tryptophan or several tyrosines per molecule can be detected by tryptophan/tyrosine fluorescence after capture using a tethered peptide (produced by biopanning a library of peptides). A variety of microbes, including algae, fungi, protozoans, and bacteria export exotoxins that are amenable to detection using this technology. Selected tissues of a variety of higher plants yield toxic proteins. A variety of animals, including reptiles, amphibians, marine invertebrates, scorpions, spiders, and insects, produce toxic proteins and peptides as well. The following list contains examples of toxic proteins and peptides that can be captured and detected using the technology described herein.

Ridinus communis (castor bean)	ricins
Apis mellifera (honey bee)	mellitin
Latrodectus mactans (black widow spider)	α-latrotoxin
Agelenopsis aptera (funnel web spider)	ϖ-agatoxin TK
Bunodosoma granulifera (sea anemone)	K+-channel-blocking toxin
Bungarus multicinctus (krait)	β-bungarotoxin
Naja naja atra (Formosan cobra)	cobratoxins
Bacteria:	
Bacillus anthracis	anthrax toxins (all components
Clostridium botulinum	botulinum toxins
Vibrio Cholerae	cholera toxin
Clostridium perfringens	α-toxin (phospholipase C)
Corynebacterium diphtheriae	diphtheria toxin
Escherichia coli	heat-labile enterotoxin
Bordetella pertussis	pertussis toxin
Shigella dysenteriae	Shiga toxin
Staphylococcus aureus	toxic shock syndrome

	toxin-1
Clostridium tetani	tetanus toxin
Yersinia pestis	YopE
Helicobacter pylori	vacuolating cytotoxin A

Examples of human bioactive peptides (including peptide hormones) that can be detected using tryptophan/tyrosine fluorescence:

adrenocorticotropic hormone (ACTH)
bombesin
gastrins
gastrin-releasing peptide (GRP)
neuropeptide Y (NPY)
luteinizing hormone releasing hormone (LH-RH)
β-melanocyte stimulating hormone
parathyroid hormone (PTH)
somatostatin
endothelins

The various ligands are preferably tethered to a substrate by means of organic coupling agents which are themselves well known to those skilled in the art. When using a glass substrate for the sensor chip, it is frequently preferred to employ, in the practice of the present invention, organosilane compounds having the following general structure:

$$\begin{array}{c} R_1 \\ | \\ R_2 - \mathrm{Si} - R_4 \\ | \\ R_3 \end{array}$$

wherein R_1 through R_3 are each selected from the group consisting of hydrogen, alkyl groups containing 1 to 6 carbon atoms, aryl groups containing 6 to 12 carbon atoms and alkoxy groups containing 1 to 4 carbon atoms, with at least one of R_1, R_2 and R_3 being an alkoxy group. R_4 is an organic group containing at least 3 carbon atoms and also containing a functional group capable of reaction with the ligand. Without limiting the invention, suitable organic groups are polyamines and polyethers containing 3 to 30 carbon atoms. Also suitable for use in the practice of the invention are coupling agents containing other functional groups such as epoxy groups, amino groups and unsaturated functional groups, OH groups, thiol groups and the like, which are capable of reaction with the various ligands. Without limiting the invention as to theory, it is believed that the ligand reacts with the functional group, preferably a terminal functional on the organosilane compound while the readily hydrolyzable alkoxy group attached directly to the silicon atom has the capability of reacting directly with the surface of the glass substrate of the sensor chips. Thus, the ligand is tethered to the surface of the glass through the coupling agent.

Thus the ligand tethered to the glass surface may be illustrated by the following:

$$\text{Glass-O-}\underset{|}{\overset{|}{\text{Si}}}\text{-(CH}_2)_3\text{-NH-(CH}_2)_3\text{-NH-(CH}_2)_5\text{-NH-(CH}_2)_{12}\text{-NH-(CH}_2)_5\text{-NJ-LIGAND}$$

The chemical reactions used in tethering ligands to the surface of the sensor chip are known to those skilled in the art and are described in the literature. Such reactions may be found in G.T. Hermanson, Bioconjugate Techniques, San Diego: Academic Press, 1966; Hansson et al., "Carbohydrate-Specific Adhesion of Bacteria to Thin Layer Chromatograms: A Rationalized Approach to the Study of Host Cell Glycolipid Receptors", Analytical Biochemistry, 146, 158-163 (1985); and, Nilsson et al., "A Carbohydrate Biosensor Surface for the Detection of Uropathogenic Bacteria", Bio/Technology, 12, 1376-1378, December 1994.

Illustrative of such reactions are those used to tether ferroxamine as a ligand to the surface of a glass sensor chip. In the first stage, a glass surface containing free hydroxyl groups is first reacted with a 2% solution of gamma-N-(aminopropyl)-gamma-aminopropyltrimethoxysilane to attach the silane to the glass surface:

$$\text{Glass-OH} + (CH_3O)_3\text{-Si-}(CH_2)_3\text{-NH-}(CH_3)_3\text{-}NH_2 \rightarrow \qquad \text{(I)}$$

$$\text{Glass-O-}\overset{|}{\underset{|}{\text{Si}}}\text{-}(CH_2)_3\text{-NH-}(CH_2)_3\text{-NH}$$

The product of that reaction can then be reacted with glutaraldehyde at a pH of about 8 to form the corresponding aldehyde:

$$\text{Glass-O-}\overset{|}{\underset{|}{\text{Si}}}\text{-}(CH_2)_3\text{-NH-}(CH_2)_3\text{-N=C-}(CH_2)_3\text{-}\overset{\overset{\text{O}}{\|}}{\text{C}}\text{-H} \qquad \text{(II)}$$

The aldehyde, in turn, can be reacted with a diamine:

$$H_2N\text{-}(CH_2)_{12}\text{-}NH_2 \qquad \text{(III)}$$

$$\text{Glass-O-}\overset{|}{\underset{|}{\text{Si}}}\text{-}(CH_2)_3\text{-NH-}(CH_2)_3\text{-N} = \underset{\underset{\text{H}}{|}}{\text{C}}\text{-}(CH_2)_3\text{-}\underset{\underset{\text{H}}{|}}{\text{C}} = \text{N-}(CH_2)_{12}\text{-}NH_2 \qquad \text{(IV)}$$

Next, the product of the preceding reaction is reacted with glutaraldehyde to introduce a (terminal) aldehyde group:

$$\text{Glass-O-}\overset{|}{\underset{|}{\text{Si}}}\text{-}(CH_2)_3\text{-NH-}(CH_2)_3N = \underset{\underset{H}{|}}{C}\text{-}(CH_2)_3\text{-}\underset{\underset{H}{|}}{C} = N\text{-}(CH_2)_{12}\text{-}N = CH\text{-}(CH_2)_3\text{-CHO} \qquad \text{(V)}$$

which can then be reduced using $NaCNBH_3$ to yield:

$$\text{Glass-O-}\overset{|}{\underset{|}{\text{Si}}}\text{-}(CH_2)_3\text{-NH-}(CH_2)_3\text{-NH-}(CH_2)_5\text{-NH-}(CH_2)_{12}\text{-NH-}(CH_2)_4\text{-CHO} \qquad \text{(VI)}$$

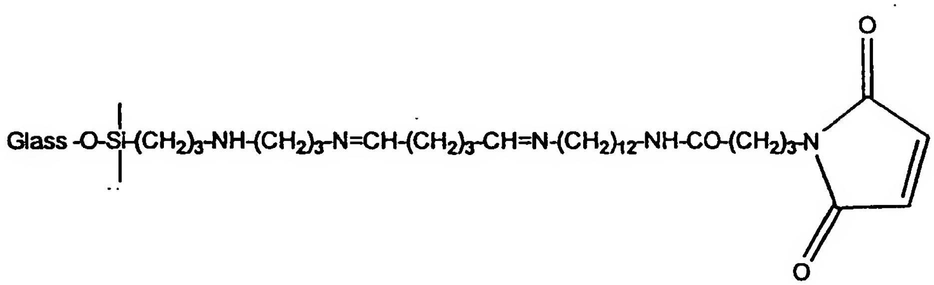
The foregoing silane coupling agent bonded to the surface can then be derivatized by reaction with deferrioxamine B (or DFA) at an alkaline pH to yield:

$$\text{Glass-O-}\overset{|}{\underset{|}{\text{Si}}}\text{-}(CH_2)_3\text{-NH-}(CH_2)_3\text{-NH-}(CH_2)_5\text{-NH-}(CH_2)_{12}\text{-NH-}(CH_2)_4\text{-CH} = \underset{\underset{H}{|}}{N}\text{-DFA} \qquad \text{(VII)}$$

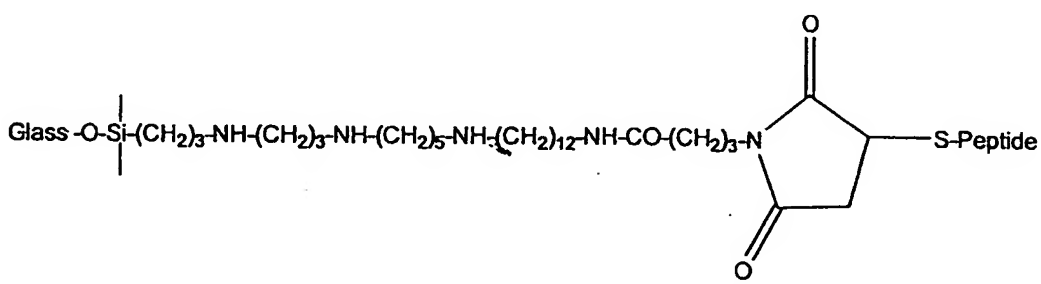
The DFA can then be complexed with Fe by reaction with a ferrous salt in aqueous medium to form the ligand.

As will be appreciated by those skilled in the art, many other techniques can likewise be used to tether an appropriate ligand to the surface of the sensor chip. For example, thiol-terminated peptides can be tethered to the surface of a glass sensor chip using similar reactions. For

example, IV above can be reacted with N-(gamma-alemidobutyryloxy) succinimide ester (GMBS) to form the following derivative:

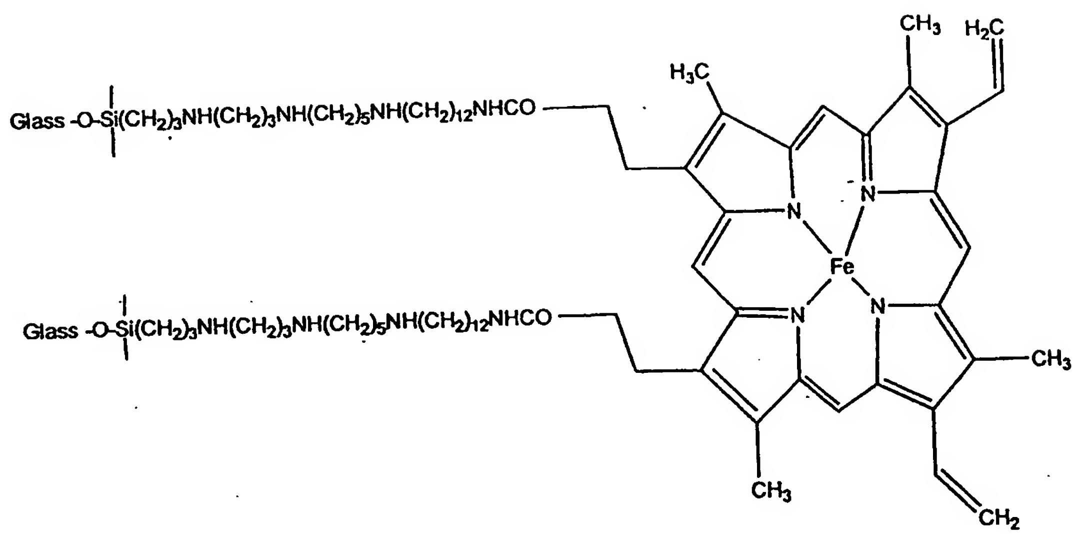


That derivative can, in turn, be sequentially reacted with the thiol-terminated peptide to form the corresponding peptide terminated compound. That, in turn, can, if desired, be reduced using sodium cyanoborohydride to yield the following peptide ligand tethered to the glass surface:



Similarly, the same overall reaction scheme can likewise be used to tether hemin to the glass surface of the sensor chip. Hemin, also known as ferriprotoprophyrin IX, can be reacted with N-hydroxysuccinimide to form the hemin di(N-hydroxysuccinimide) ester. That diester can then be reacted with the product IV referred to above which has been previously reduced with sodium cyanoborohydride to form the following

tethered ligand:



Thus, as described above, a different ligand is tethered to each of the sections of the sensor chip. The sensor chip is then contacted with a sample containing an unknown organism, protein or peptide whereby specific ligands on the surface of the chip bind to specific analytes, selectively capturing them. The sensor chip is then subjected to electromagnetic radiation using the equipment described in Serial No. 659,043 so that each section of the sensor chip is exposed to an appropriate wavelength of electromagnetic radiation to excite fluorescence characteristic of the presence of bound analytes. Appropriate fluorescence signals generated by metabolites or other biomolecules specific to pathogenic microorganisms on exposure to electromagnetic radiation are set

forth in Table 2. Note NAD[P]H is only present in viable or respiring cells; calcium dipicolinate as a significant presence in spores of the order of about 15% but is otherwise rare in nature. The relative signals as measured in bacteria and spores are shown in Figs. 3 and 4, respectively. Any proteinaceous toxin or peptide hormone that contains tryptophan or tyrosine will also generate fluorescence signals as noted in Table 2 for these fluorophores.

The sensor is illustrated in Fig. 5 of the drawing in schematic form, and includes a source of light 10 having the desired wavelength. As will be appreciated by those skilled in the art, the source of electromagnetic radiation 10 can be any of a variety of devices producing UV light having a wavelength less than about 400 nm. For example, the source of electromagnetic radiation 10 can be a laser or various types of lamps emitting electromagnetic radiation generally within the UV range.

The wavelength of light is chosen by an excitation filter 12, preferably in the form of a narrow band-width filter passing electromagnetic radiation having wavelengths within the desired range. For example, when seeking to excite NADH, the filter 12 should be one which emits electromagnetic radiation

within the range of about 350 to 390nm and preferably with a peak of about 366nm.

Positioned to receive the light emitted from the light source 10 through filter 12 is a conduit for electromagnetic radiation 14, preferably in the form of a fiber optic element or bundle of elements, capable of conducting the electromagnetic energy passing from the source 10 through the filter 12. The fiber optic bundle terminates in a probe 16, which can be a probe used to successively scan each of the sections of the sensor chip, as in a rastor scan or in one element of a multi-element probe system with one element for each section.

Also contained within the probe are a pair of additional fiber optic elements or bundles 20 and 22 illustrated in Fig. 2, positioned to transmit electromagnetic radiation from the sensor chip 8 for detection. In the illustrated embodiment, fiber optic element 20 conveys electromagnetic energy from the sensor chip 8 to an emission filter 24; that filter is chosen to pass electromagnetic radiation having the wavelength of the fluorescence generated in the microbial cells, peptides or proteins present on each of the sections of the sensor chip 8.

It is also desirable, in the practice of the

invention, to use another filter 26 associated with fiber optic element 22 which passes only electromagnetic radiation having substantially the same wavelength as the electromagnetic radiation directed to the sensor chip through fiber optic element 14. Thus, filter 26 should pass electromagnetic radiation within the same range as that used for excitation. The choice of excitation and emission filters is set forth in Table 2. The filter combinations can be easily changed by mounting the filters on a rotating wheel or sliding mechanism to provide detection of all of the compounds in Table 2.

The apparatus shown in Fig. 5 also includes a pair of detector elements 28 and 30 which detect electromagnetic radiation passing through filters 24 and 26, respectively. As will be appreciated by those skilled in the art, the detectors 28 and 30 are elements sensitive to electromagnetic radiation, converting that radiation into an electrical signal which is proportional to the intensity of the radiation presented to the detectors through filters 24 and 26, respectively. Conventional devices including photodiodes, photomultiplier, tubes, video cameras, charge-coupled devices as well as other detectors may be used.

Because the electromagnetic radiation passed through

the associated filter 24 is essentially limited to the wavelength of the electromagnetic energy associated with the fluorescence of a specific metabolite or other biomolecules, the amount of fluorescence due to that metabolite passing through the filter 24 from the sensor chip 8 and detected by the fluorescence detector 28 is related to the amount of that metabolite present and thus to the number of microorganism, protein molecules or peptide molecules present. The electromagnetic radiation passed through fiber optic element 22 is filtered to pass only electromagnetic radiation having a wavelength substantially the same as that of the electromagnetic energy directed into the sensor chip for that particular section; detector 30 measures only the reflected radiation from the sensor chip. Both detectors 28 and 30 thus convert the electromagnetic energy to a corresponding electrical signal, and the signal indicative of the presence of analytes of interest is determined by dividing the amount of electromagnetic radiation passing through filter 24 to detector 28 (representing the fluorescence of any captured species present on that section of the sensor chip) by the electromagnetic radiation passing through filter 26 to the detector 30 (representing the reflected electromagnetic radiation). This signal is then normalized by subtraction of this ratio for the blank section of the sensor chip. Thus, the signal may be represented by the difference

between the fluorescence divided by the reflectance for a section containing a captured analyte of interest minus the fluorescence divided by the reflectance for a blank section.

An alternate system for scanning the sensor chip 8 is illustrated in Fig. 2 of the drawing showing a sensor chip 8 which has been contacted with an analyte. Various sections 9 contain no captured analyte thereon while other sections 7 do have a captured analyte. It is possible, and sometimes desirable, to determine the presence of a bound analyte in each of the sections of the sensor chip 8 simultaneously.

That can be done using a system described in Fig. 2 having a matrix support member 40 which includes a plurality of probes 16 mounted therein, with the probes 16 mounted in the matrix 40 being patterned to correspond to the pattern of sections in the chip 8.

In the preferred embodiment, the matrix 40 includes one probe 16 for each of the sections on the sensor chip 8, although, as will be understood by those skilled in the art, either fewer or greater probes may be used under some circumstances. The matrix is then positioned proximate to a chip 8 which has been exposed to an unknown sample and each of

the sections is exposed to electromagnetic radiation simultaneously. Accordingly, each of the sections is simultaneously examined by electromagnetic radiation to detect the presence of captured analytes on each section of the chip 8. The system of Fig. 2 otherwise operates in the same manner as that described in Fig. 5.

By comparing fluorescence signals to reflected signals, the system as described in detail in the foregoing, copending application, normalizes the signals. That allows the system to compensate for variations in the distance of the probe from the surface of the chip 8 and variations between different surfaces. As will be appreciated by those skilled in the art, it is possible, and sometimes desirable, to either use multiple sources of electromagnetic radiation or to employ multiple filters 12. In that way, the electromagnetic radiation directed to a single section of the sensor chip 8 may be changed going from one section of that chip to another section of the chip. That technique thus permits different wavelengths of electromagnetic radiation to be directed to different sections of the sensor chip, depending on the ligand tethered to that particular section of the sensor chip.

As will be appreciated by those skilled in the art, a

variety of bacterial cell components or metabolites as well as proteins and peptides exhibit intrinsic fluorescence when illuminated by UV light. For example, NADH has been extensively used in the study of various organisms and can be employed to determine whether a particular microbial sample contains viable or respiring cells (Fig. 3). Similarly, tryptophan likewise exhibits fluorescence indicative of spores, nonviable cells, most protein toxins and many peptide hormones, while calcium dipicolinate exhibits fluorescence indicative of the presence of spores (Fig. 4). Those fluorophores, their excitation and emission frequencies in vivo as well as the indications they provide are set forth in Table 2:

Fluorophore	Excitation (nm) Range Peak	Emission (nm) Range Peak	Source
NAD[P]H	350-390 366	415-465 440	Viable [Respiring] Cells
tryptophan (tyrosine)	265-295 280 (274)	315-385 350 (295-315) (305)	Spores Non-Viable Cells Most Exotoxins Many Peptides
Ca++dipicolinate	280-310 295	380-460 420	Spores

In the practice of the present invention, a sample containing unknown microbes can be contacted with the sensor chip whereby one or more receptors of the bacteria react with

various different ligands tethered to the various sections of the chip. Then, the fluorescence of the chip can be measured with the probe 16 for the purpose of detecting which of the sections of the sensor chip have analytes bonded thereto. As examples, Myobacterium siderophores can be used to capture mycobacteria such as mycobacterium tuberculosis. Helicobacter pylori can be captured using tethered N-acetylneuroaminyl-alpha-2,3-galactose. The peptide:

GADRSYLSFIHLYPELAGAC

can be tethered, by means of the terminal systeine group to suppress capture Staphylococcus aureus toxic-shock toxin-1.

As indicated above, some of the analytes of interest can be identified by determining the presence of a single captured microorganism, protein or peptide. In other cases, however, a series of two or more captured analytes of interest is indicative of the identity of a particular analyte. As an example, consider a sensor chip having an area of three sections along the horizontal axis and three sections along the vertical axis as illustrated below:

A1	A2	A3
B1		
C1		

As an example, the sections identified can be provided with the following ligands tethered to each specific section as set forth in the following table:

Section Location	10x10 Array Ligand
A1	asialo G_{m1}
A2	hemin
A3	pyochelin
B1	GalNAcβGal
B2	alcaligin
B3	fibronectin peptide
C1	diferric transferrin
C2	staphyloferrin
C3	ferrioxamine B

It has been found that Pseudomonas aeruginosa can be identified as the microorganism when analytes are detected in sections A1, A2, A3, B1, C1 and C3. Similarly, Klebsiella Pneumoniae is detected when sections A2, B1, C1 and C3 have analytes captured thereon, and Serratia marescens is identified when sections A2 and C3 have analyte captured thereon. Similarly, Staphylococcus aureus can be identified when sections A2, B1, B3, C2 and C3 contain analyte captured thereon.

It will be understood that various changes and modifications can be made in the determination, procedure, formulation and use without departing from the spirit of the invention, especially as defined in the following claims.

<u>What Is Claimed</u>:

1. A method for the taxonomic identification of microorganisms comprising:

(a) contacting a microorganism-containing sample with a sensor chip, said sensor chip having a patterned array on the surface thereof containing a plurality of sections, with each section having bonded thereto a ligand capable of binding thereto a microorganism;

(b) directing electromagnetic radiation onto the surface of the sensor chip to ascertain the presence of a microorgansim bound to te ligand on each section; and

(c) taxonomically identifying the microorganism contained in the sample as a function of the combination of different ligands having a microorganism bound thereto.

2. A method as defined in cliam 1 wherein each ligand is tethered to the surface of the chip by means of a

coupling agent.

3. A method as defined in claim 1 wherein the coupling agent is an organosilane compound.

4. A method as defined in claim 1 wherein the sensor chip is formed with a glass surface.

5. A method as defined in claim 1 wherein the chip has a surface formed of a non-reactive plastic.

6. A method as defined in claim 1 wherein the ligands are selected from the group consisting of heme compounds, siderophores, oligosaccharides and peptides.

7. A method as defined in claim 1 wherein the elecromagnetic radiation has a wavelength less than about 400 nm to detect NAD[P]H as an indication of the presence of respiring cells.

8. A method as defined in claim 1 wherein the electromagnetic radiation has an excitation wavelength within the range of about 265 to 295 nm to detect the presence of trypotophan as an indication of the presence of spores,

nonviable cells, exotoxins and peptides.

9. A method as defined in claim 1 wherein the electromagnetic radiation has an excitation wavelength within the range of about 280 to about 310 nm to detect calcium dipicolinate as an indication of the presence of spores.

10. A method for the identification of microorganisms, proteins and peptides comprising:

(a) contacting an analyte-containing sample with a sensor chip having a patterned array on the surface thereof containing a plurality of sections, with each section having bonded thereto a ligand capable of binding a microorganism, protein or peptide;

(b) directing the electromagnetic radiation onto the surface of the sensor chip to ascertain the presence of a microorganism, protein or peptide bound to the ligand of each section; and

(c) identifying the microorgansim, protein or peptide contained in the sample as a function of the

combination of different ligands having a microorgansim, protein or peptide bound thereto.

11. A method as defined in claim 10 wherein the analyte contains bacteria, viruses, protozoans, algae and fungi.

12. A method as defined in claim 10 wherein each ligand is tethered to the surface of the chip by means of a coupling agent.

13. A method as defined in claim 10 wherein the coupling agent is an organosilane compound.

14. A method as defined in claim 10 wherein the ligands are selected from the group consisting of heme compounds, siderophores, oligosaccarides and peptides.

15. A sensor chip for use in the identification of microorganisms, proteins and peptides comprising:

(a) a substrate having a biocompatible, nonreactive surface having a patterened array of sections thereon; and

(b) a plurality of ligands capable of capturing different microorganisms, proteins and peptides tethered to the surface of the substrate, with different ligands being present on sections of the substrate.

16. A chip as defined in claim 15 wherein each ligand is tethered to the surface of the chip by means of a coupling agent.

17. A chip as defined in claim 16 where the coupling agent is an organosilane.

18. A chip as defined in claim 15 wherein the surface of the chip is a glass surface.

19. A chip as defined in claim 15 wherein the ligands are each selected from the group consisting of heme compounds, siderophores, oligosaccarides, and peptides.

20. Apparatus for the identification of microorgansims, proteins and peptides using a sensor chip having a patterned array of sections thereon and a plurality of ligands tethered to the surface of the chip, with sections having

different ligands tethered thereto, comprising:

(a) means for directing electromagnetic radiation toward at least one of the sections of the chip to determine if that one section has a microorganism, protein and/or peptide captured thereto;

(b) at least one detector for electromagnetic radiation capable of converting the radiation into an electrical signal; and

(c) means for receiving electrical signals corresponding to the emission of electromagnetic radiation from said one section and electrical signals corresponding to the electromagnetic radiation reflected by said one section as a measure of the amount of microorgansim, protein and/or peptide captured on said one section.

21. Apparatus as defined in claim 20 wherein the means for directing electromagnetic radiation is adapted to direct electromagnetic radiation of at least two discrete bands of wavelengths.

22. Apparatus as defined in claim 20 wherein the means for directing electromagnetic radiation includes at least two excitation filters.

23. Apparatus as defined in claim 20 wherein the means for directing electromagnetic radiation simultaneously directs said radiation toward a plurality of sections simultaneously.

24. Apparatus as defined in claim 20 wherein the means for directing electromagnetic radiation directs said radiation sequentially toward each of the sections of the chip.

FIG.1

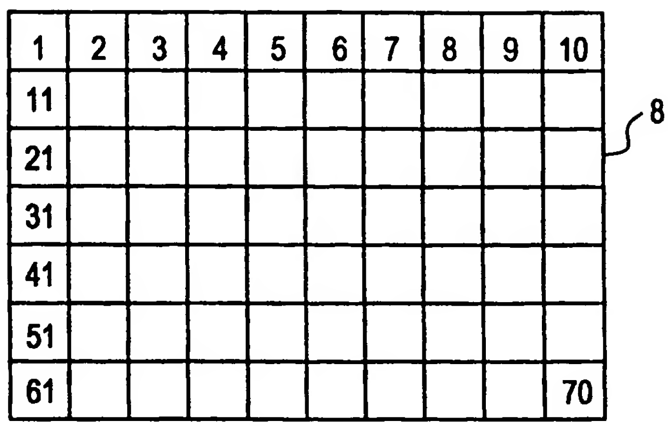


FIG.2

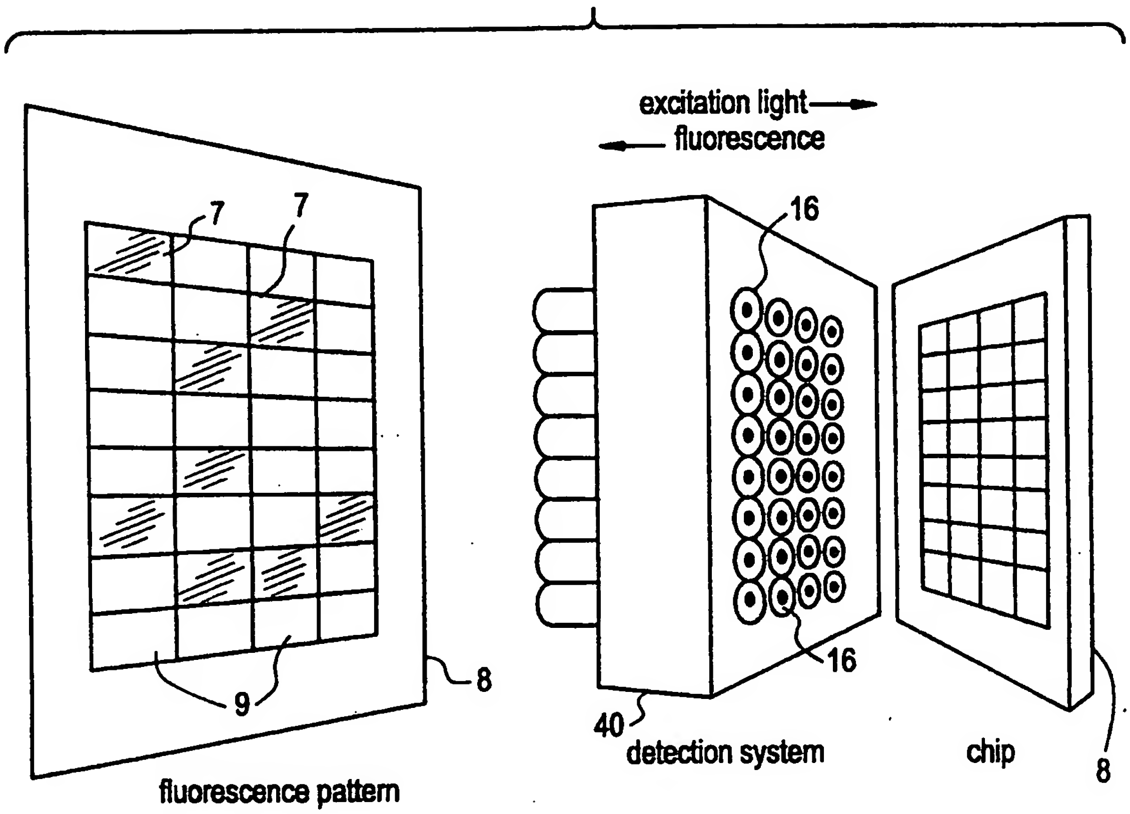

excitation light
fluorescence
7
7
16
16
9
8
40
detection system
chip
8
fluorescence pattern

FIG.3

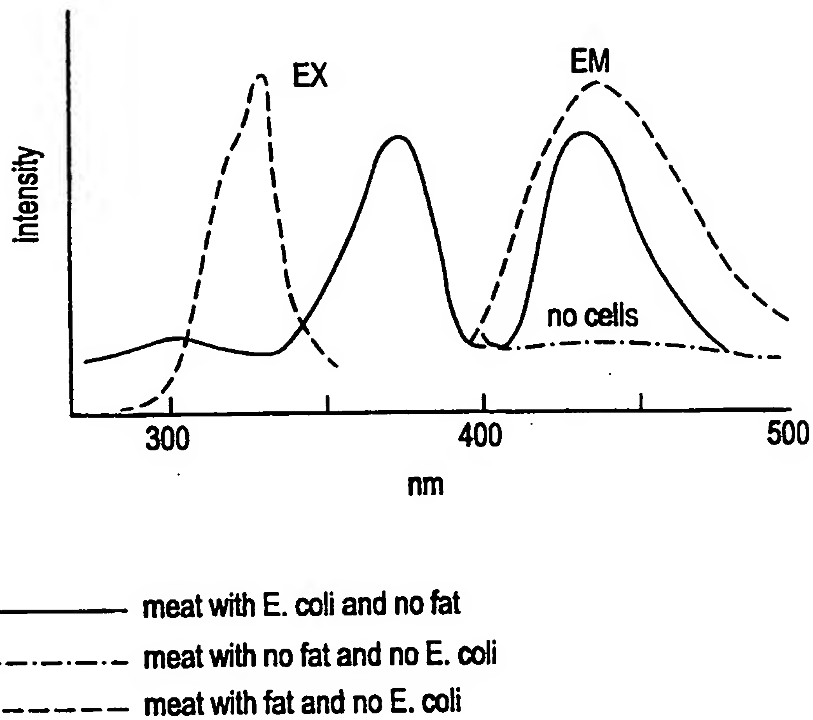

EX
EM
intensity
no cells
300
400
500
nm
meat with E. coli and no fat
meat with no fat and no E. coli
meat with fat and no E. coli

FIG.4

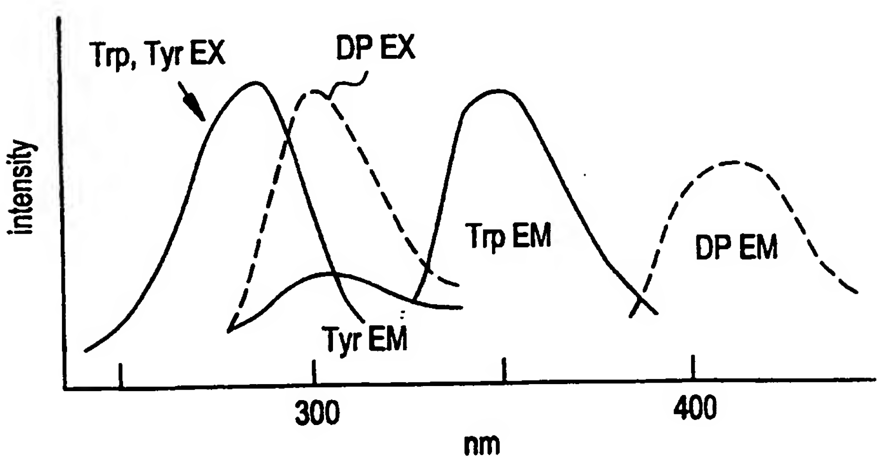
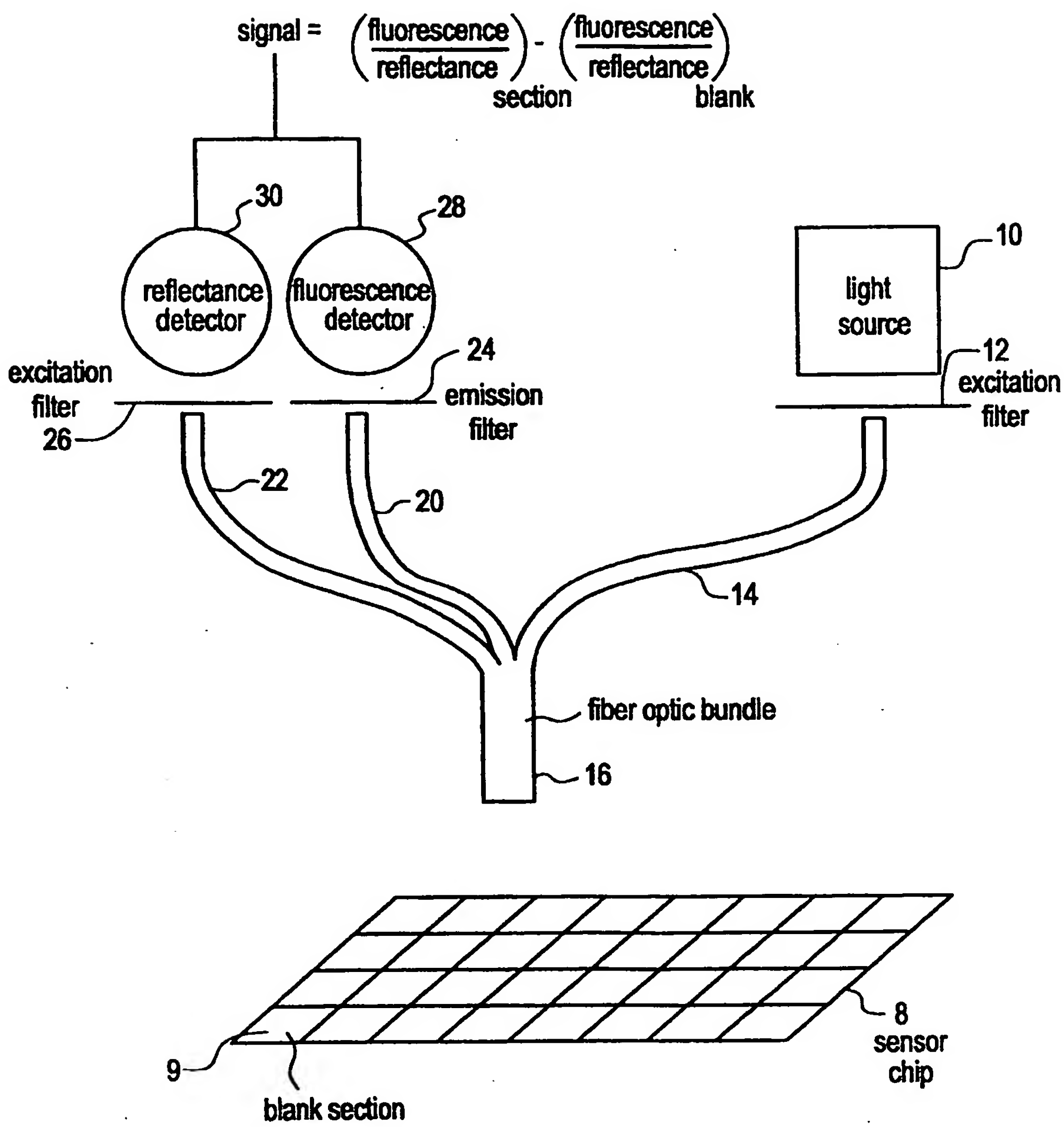

Trp, Tyr EX
DP EX
intensity
Trp EM
DP EM
Tyr EM
300
400
nm

FIG.5


signal = (fluorescence / reflectance) section - (fluorescence / reflectance) blank
30
28
reflectance detector
fluorescence detector
10
light source
excitation filter
26
24
emission filter
12
excitation filter
22
20
14
fiber optic bundle
16
8
sensor chip
9
blank section

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/08458

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/543 G01N33/569 G01N33/68 G01N21/64

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C12Q C12M

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 276 968 A (YELLOWSTONE DIAGNOSTICS CORP) 3 August 1988 see the whole document	1-24
A	WO 94 24561 A (NILSSON & MANDENIUS) 27 October 1994 see abstract	1,10,15,20
A	US 5 474 910 A (ALFANO ROBERT R) 12 December 1995 see the whole document	1,10,15,20
A	US 4 631 413 A (JENSEN SVEND A K ET AL) 23 December 1986 see the whole document	1,10,15,20

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 July 1998	04/08/1998

Name and mailing address of the ISA	Authorized officer
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2

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,A	WO 97 46867 A (B E SAFE INC ;POWERS LINDA (US)) 11 December 1997 cited in the application ---	1,10,15, 20
P,A	WO 97 27316 A (UNIV CALIFORNIA ;CHARYCH DEBORAH (US); NAGY JOHN (US)) 31 July 1997 see the whole document -----	1,10,15, 20

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 98/08458

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
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			US	4886761	A	12-12-1989
			AU	604830	B	03-01-1991
			AU	1099588	A	04-08-1988
			CA	1305921	A	04-08-1992
			JP	63277969	A	15-11-1988
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US 4631413	A	23-12-1986	SE	451163	B	07-09-1987
			SE	455645	B	25-07-1988
			AU	566669	B	29-10-1987
			AU	2913284	A	20-12-1984
			DE	3473797	A	06-10-1988
			DK	287584	A,B	14-12-1984
			EP	0128889	A	19-12-1984
			JP	1680947	C	31-07-1992
			JP	3040820	B	20-06-1991
			JP	60040939	A	04-03-1985
			SE	8303327	A	14-12-1984
			SE	8304288	A	06-02-1985
WO 9746867	A	11-12-1997	US	5760406	A	02-06-1998
			AU	3294897	A	05-01-1998
WO 9727316	A	31-07-1997	AU	1842297	A	20-08-1997